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Characterization of the *p*-Toluenesulfonate Operon *tsaMBCD* and *tsaR* in *Comamonas testosteroni* T-2

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Comamonas testosteroni **T-2 uses a standard, if seldom examined, attack on an aromatic compound and oxygenates the side chain of *p*-toluenesulfonate (TS) (or *p*-toluenecarboxylate) to *p*-sulfobenzoate (or terephthalate) prior to complete oxidation. The expression of the first three catabolic enzymes in the pathway, the TS methyl-monooxygenase system (comprising reductase B and oxygenase M; TsaMB), *p*-sulfobenzyl alcohol dehydrogenase (TsaC), and *p*-sulfobenzaldehyde dehydrogenase (TsaD), is coregulated as regulatory unit R1 (H. R. Schläfli Oppenberg, G. Chen, T. Leisinger, and A. M. Cook, Microbiology [Reading] 141:1891–1899, 1995). The components of the oxygenase system were repurified, and the N-terminal amino acid sequences were confirmed and extended. An internal sequence of TsaM was obtained, and the identity of the [2Fe-2S] Rieske center was confirmed by electron paramagnetic resonance spectroscopy. We purified both dehydrogenases (TsaC and TsaD) and determined their molecular weights and N-terminal amino acid sequences. Oligonucleotides derived from the partial sequences of TsaM were used to identify cloned DNA from strain T-2, and about 6 kb of contiguous cloned DNA was sequenced. Regulatory unit R1 was presumed to represent a four-gene operon (*tsaMBCD*) which was regulated by the LysR-type regulator, TsaR, encoded by a deduced one-gene transcriptional unit. The genes for the inducible TS transport system were not at this locus. The oxygenase system was confirmed to be a class IA mononuclear iron oxygenase, and class IA can now be seen to have two evolutionary groups, the monooxygenases and the dioxygenases, though the divergence is limited to the oxygenase components. The alcohol dehydrogenase TsaC was confirmed to belong to the short-chain, zinc-independent dehydrogenases, and the aldehyde dehydrogenase TsaD was found to resemble several other aldehyde dehydrogenases. The operon and its putative regulator are compared with units of the TOL plasmid.**

Aromatic sulfonates are produced in the multimillion tonne range annually and dispersed in the environment as detergents, dyestuffs, and additives to products as diverse as ink and engine oil (24). These charged compounds are now readily found in the environment (see, e.g., reference 38); defined, naturally occurring sulfonates, usually aliphatic compounds, are also known (64; see also reference 76). At least five different mechanisms for the desulfonation of the aromatic compounds in aerobes are known (17, 21, 34, 39, 84; see also reference 16), and different organisms can have different strategies to attack, e.g., *p*-toluenesulfonate as a carbon source (21, 34, 61). There is no information on the genetics of these pathways, and in only one case is an extensive understanding of the enzymology and physiological controls available, the degradation of *p*-toluenesulfonate by *Comamonas testosteroni* T-2 (35, 42, 61).

C. testosteroni T-2, grown with toluenesulfonate as the sole source of carbon and energy, synthesizes an inducible, specific, secondary proton symport system (42) and inducible enzymes that convert toluenesulfonate (or toluenecarboxylate) to sulfobenzoate (or terephthalate) in three steps (Fig. 1) (41). These reactions require four enzymes, now termed TsaMB (*p*-toluenesulfonate methyl-monooxygenase system, comprising reductase B and oxygenase M), TsaC (*p*-sulfobenzyl alcohol dehydrogenase) and TsaD (*p*-sulfobenzaldehyde dehydrogenase), which are synthesized in one regulatory unit, termed R1 (61).

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The pathway (Fig. 1) bears superficial comparison with the upper pathway of the TOL plasmid (see, e.g., reference 3) and is thus representative of a major mode of attack on aromatic compounds: degradation initiated at the alkyl side chain (41). The superficiality is immediately visible at the enzyme level. Oxygenase TsaMB (strain T-2) has been attributed to the class IA multicomponent, mononuclear iron oxygenases (5, 44, 51), whereas oxygenase XylMA (TOL plasmid) belongs to a class of multicomponent, di-iron monooxygenases (65, 70); the alcohol dehydrogenases seem to belong to different classes (41, 66), and the same was believed to be true for the aldehyde dehydrogenases (41).

Whereas it is reasonable to hypothesize that regulatory unit R1 (Fig. 1) (61) represents an operon (see, e.g., references 74 and 83), it is impossible to anticipate the type of regulation involved, e.g., LysR-, XylS/AraC-, or XylR-type (33, 53, 59). In addition, the (presumptive) genes encoding the inducible transport system for toluenesulfonate may be an integral part of an operon encoding degradative enzymes (49, 80) located close to the degradative operon (52) or found at another locus (48). The transport system for toluenesulfonate does not transport toluenecarboxylate (42).

We now report more information, including two enzyme purifications (TsaC and TsaD), on the four enzymes of regulatory unit R1 in *C. testosteroni* T-2 (Fig. 1) (61), and we report that R1 is indeed an operon comprising *tsaMBCD*, which is presumably under the control of a putative transcriptional LysR-type regulator, TsaR. The genes encoding the transport system(s) are distant from this structure. This is the first report characterizing both the enzymes and the nucleotide sequence encoding the attack on the side chain of an aromatic compound.

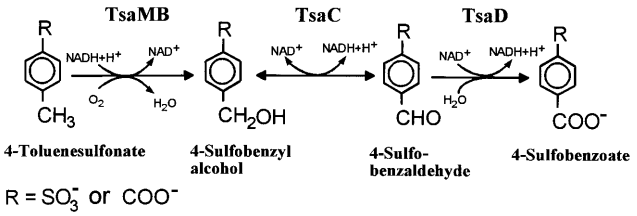


FIG. 1. The reactions catalyzed by the monooxygenase system TsaMB, the alcohol dehydrogenase TsaC, and the aldehyde dehydrogenase TsaD of regulatory unit R1 (61) of *C. testosteroni* T-2.

MATERIALS AND METHODS

Bacteria, growth conditions and plasmids. *C. testosteroni* T-2 (DSM 6577) was grown in batch culture at 30°C in mineral salts medium, pH 7.2, containing 6 mM toluenesulfonate (40). Most cells for enzyme purifications were grown in a 12.5-liter fermentor with an 8- to 10-liter working volume (Biostat V; Braun, Melsungen, Germany), harvested early (60 mg of protein/liter, to avoid problems with foaming and clumping) in a Pellikon cassette filtration system (Millipore, Neu-Isenburg, Germany), washed by centrifugation, and stored frozen, as in our earlier work (39). When vanillate or *p*-methoxybenzoate (6 mM) was used as the carbon source, the pH was adjusted to 7.0.

Escherichia coli DH5α was used as the host for pBluescript-KSII constructs and was routinely grown in Luria broth containing 100 μg of ampicillin per ml at 37°C.

Preparation of cell extracts, enzyme assays, and enzyme purifications. Cells were disrupted in a French pressure cell, and the crude extract, in 20 mM phosphate buffer (pH 7.0) with or without 1 mM dithiothreitol, was prepared as described elsewhere (39). Reductase TsaB was assayed at 25°C by the reduction of cytochrome *c* (39). Monooxygenase TsaM was assayed by oxygen uptake in a coupled assay with the reductase (40). Each component (TsaB and TsaM) was purified in two chromatographic steps, anion exchange and hydrophobic interaction (40).

Cell extract for the purification of alcohol dehydrogenase TsaC and aldehyde dehydrogenase TsaD was prepared without dithiothreitol. Activities were assayed at room temperature (21°C) by oxidation (TsaD) or reduction (TsaC) of *p*-carboxybenzaldehyde as described elsewhere (41). TsaC was purified in two chromatographic steps (anion exchange and hydrophobic interaction), essentially as described elsewhere (41). A new purification scheme for TsaD was developed, involving an anion exchange and subsequent gel filtration. The anion-exchange column (Mono Q HR 10/10; Pharmacia, Uppsala, Sweden) was equilibrated with 20 mM phosphate buffer, pH 6.8 (buffer A), and proteins were eluted with an increasing gradient of sodium sulfate (to 1 M, buffer B) at a flow rate of 4 ml/min as follows: from 0 to 40 min, 100% buffer A; from 40 to 68 min, 0 to 3% buffer B; from 68 to 130 min, 3 to 6% buffer B; from 130 to 200 min, 6 to 20% buffer B. Four-milliliter fractions were collected, and fraction 17 was worked up. In the second purification step the gel filtration column (Superose 12 HR 10/30; Pharmacia) was equilibrated with 150 mM sodium sulfate in 50 mM phosphate buffer, pH 6.8, at a flow rate of 0.5 ml/min and a sample volume of 300 μl was used.

Only the fraction of TsaC or TsaD with the highest specific activity was used for further work. Purifications were monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie staining (37, 40). Purified protein bands were blotted to a polyvinylidenefluoride membrane (Immobilon-P; Millipore, Bedford, Mass.) and subjected to Edman degradation (39, 40).

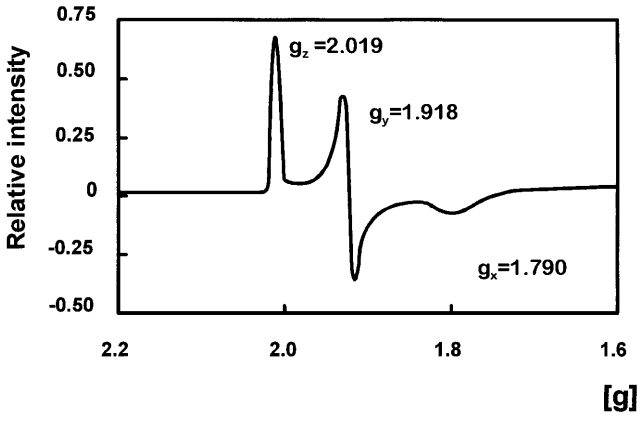


FIG. 2. EPR spectrum of the oxygenase component TsaM of monooxygenase system TsaMB from *C. testosteroni* T-2.

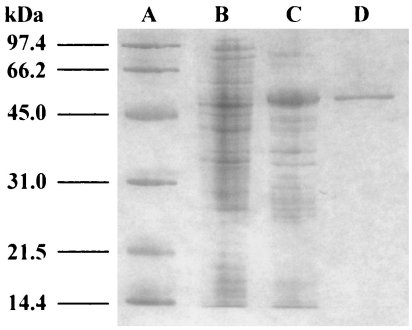


FIG. 3. SDS-PAGE, with Coomassie blue staining, of the products of purification of *p*-sulfobenzaldehyde dehydrogenase (TsaD) from *C. testosteroni* T-2. Lane A, molecular weight markers; lane B, crude extract (40 μg of protein); lane C, fraction from the anion exchanger (7 μg of protein); lane D, fraction from the gel filtration column (1 μg of protein).

Protein samples were concentrated by membrane filtration (Centricon-10; Amicon, Danvers, Mass.). The hydrophobic TsaD bound extensively to the filter unit unless the unit was first incubated overnight in 5% aqueous Tween 20 solution and then rinsed thoroughly with water.

Determinations of the molecular weights under native conditions were done with the gel filtration column. The sample volume for molecular weight markers (40) and samples was 300 μl.

EPR. Electron paramagnetic resonance (EPR) spectra of monooxygenase TsaM (about 2 mg in 0.3 ml) in the X band were recorded with an EPR 300 spectrometer (Bruker, Rheinstetten, Germany) at 10 K as described elsewhere (55).

Cyanogen bromide cleavage. Purified TsaM was separated on an SDS–12% PAGE gel, visualized by the Zn^{2+} reverse-staining method (18), and cut from the gel. The gel chip was minced, suspended in 500 μl of 0.1% CNBr, flushed with nitrogen, and allowed to react in the dark for 20 h. The reaction mixture was incubated for 2 h at 37°C with extraction solution (50% formic acid, 25% acetonitrile, 15% propanol, 10% H_2O) and then centrifuged (12,000 × *g*, 10 min). The supernatant fluid was taken to dryness in a vacuum centrifuge. The digest was dissolved in water, and protein fragments were separated by reversed-phase chromatography (ProRPC HR 10/10; Pharmacia). The column was equilibrated with 0.1% trifluoroacetic acid (0.5 ml/min), and the sample was eluted with an increasing gradient of methanol (0 to 100% in 50 min). The fragments eluted in two major peaks. All protein fragments of peak two were pooled and dried in a vacuum centrifuge. The pellet was dissolved in loading buffer and separated by SDS-PAGE in a 15% gel. Peptides were then stained with Coomassie brilliant blue. Three bands (14, 11.5, and 9.0 kDa) were identified, blotted onto a polyvinylidenefluoride membrane, and subjected to Edman degradation (see above). The N-terminal amino acids of the 9.0-kDa fragment were used to deduce an oligonucleotide probe.

Enzymes and reagents. All restriction enzymes, Klenow fragments, T4 DNA ligases, and polynucleotide kinases were obtained from Fermentas, Vilnius, Lithuania. [α-^{35}S]dATP for sequencing and [γ-^{32}P]dATP for oligonucleotide labeling were obtained from Dupont (Regensdorf, Switzerland).

All reagents for growth media and protein purification were purchased from Fluka (Buchs, Switzerland).

Preparation of total DNA and plasmid DNA. Total DNA from a 100-ml batch culture (200 mg of protein/liter of culture) of toluenesulfonate-grown *C. testosteroni* T-2 was prepared by the cetyltrimethylammonium bromide precipitation method (4). Plasmid DNA from *E. coli* was isolated with Nucleobond AX-100 columns (Macherey-Nagel, Oensingen, Switzerland).

DNA cloning, screening, sequencing, and analysis. Total DNA from strain T-2 was digested with restriction enzymes and separated in a 1% agarose gel. The DNA was blotted onto a Hybond-N nylon membrane (Amersham International, Amersham, United Kingdom). Degenerate oligonucleotide probes were 5′ labeled with [γ-^{32}P]dATP, and DNA fragments were labeled with [α-^{35}S]dATP by random oligonucleotide primers (4). Both probes were used for screening. DNA fragments were cut from the agarose gel and eluted by phenol-chloroform extraction (4). The eluted fragments were ligated into pBluescript-KSII and introduced via electroporation (4) into *E. coli* DH5α. All cloning was done with pBluescriptII.

Nucleotide sequencing of both strands was performed by the modified dideoxy chain termination method (57, 63) from double-stranded DNA templates with Sequenase (U.S. Biochemical Corp., Cleveland, Ohio), and PCR-based sequencing was performed with fluorescence-labeled nucleotides (Microsynth, Balgach, Switzerland). Nucleic acid and amino acid sequences were analyzed by using the

TABLE 1. Purification of *p*-sulfobenzyl alcohol dehydrogenase TsaC from *C. testosteroni* T-2

Purification step	Vol (ml)	Protein (μg)	Activity (nkat)	Sp act (kat/kg)	Yield (%)	Purification (fold)
Crude extract	2	45,000	6.5	0.15	100	1
Anion exchanger	4	1,300	1.8	1.4	27	9.8
Hydrophobic interaction	0.5	20	0.26	13.3	4	92

TABLE 2. Purification of *p*-sulfobenzaldehyde dehydrogenase TsaD from *C. testosteroni* T-2

Purification step	Vol (ml)	Protein (μg)	Activity (μkat)	Sp act (mkat/kg)	Yield (%)	Purification (fold)
Crude extract	2	70,700	140	2	100	1
Anion exchanger	4	648	124	192	88	96
Gel filtration	0.5	12.5	6	501	4	250

Genetics Computer Group program package (University of Wisconsin, Madison, Wis.). The evolutionary tree was generated with the CLUSTAL W program (71).

Nucleotide sequence accession number. The sequence data are available in the NCBI GenBank library under accession number U32622.

RESULTS

Biochemical characterizations and development of oligonucleotide probes for *tsaMB*. Both components of oxygenase TsaMB were repurified, and the molecular weights of the denatured proteins (43,000 and 36,000) were confirmed (40). The N-terminal amino acid sequences were confirmed and extended as follows: TsaM, MFIRNXWYVAAWDTEIPAEGL FHR; TsaB, ADVPVTVAAVRAVARDVLALELRHANGQ. The sequence of TsaM contained a suitable sequence of amino acids with low degeneracy of coding. The sequence of TsaB did not, and the amino acid composition (39), largely amino acids with high degeneracy of coding, implied that no such sequence could be expected. We therefore sequenced an internal fragment of TsaM to be able to derive a second oligonucleotide probe: $TsaM_{int}$, KPGYIHYQANYKLIVDNLLDFTHLAXV HPT.

The flavin content of the reductase component, TsaB, was consistent with the yellow color of the protein, and the ability of the protein to reduce cytochrome *c* indicated its [2Fe-2S] ferredoxin component (see reference 39). The nature of the [2Fe-2S] center in monooxygenase TsaM was presumed from its UV spectrum to be a Rieske center. We have now used EPR spectroscopy (Fig. 2; Table 4) to confirm the presence of the Rieske [2Fe-2S] center (see reference 40).

Monooxygenase TsaMB has a broad substrate range which includes *p*-methoxybenzoate (40). The monooxygenase systems vanillate-demethylase (VanAB) and *p*-methoxybenzoate-demethylase (MbdAB) reported in the literature (6, 9) are closely related to TsaMB (see below), and *C. testosteroni* T-2 utilized both *p*-methoxybenzoate and vanillate as sole sources of carbon for growth. Extracts of toluenesulfonate-grown cells of strain T-2, however, could not oxygenate vanillate, so monooxygenase system TsaMB is not identical with the vanillate-demethylase synthesized by this organism. Similarly, extracts of *p*-methoxybenzoate-grown cells of strain T-2 could not convert toluenesulfonate to sulfobenzoate, so, because TsaMB is expressed concomitantly with the dehydrogenases yielding the latter compound (61), monooxygenase system TsaMB is not identical with the *p*-methoxybenzoate-demethylase in this organism.

Purification of alcohol dehydrogenase TsaC to give identification tags. TsaC was purified to apparent homogeneity in a two-step procedure which gave a 92-fold purification (Table 1). The N terminus was unambiguously determined to be MN LNKQVAIV. SDS gel electrophoresis and gel filtration chromatography confirmed earlier data indicative of a dimeric native protein with a subunit molecular weight of 29,000 (see reference 41).

Purification of aldehyde dehydrogenase TsaD to give identification tags. TsaD was extensively purified (Fig. 3) in a two-step procedure which gave a 250-fold purification (Table 2). The keys to this purification were treatment of the concentration membranes to eliminate extensive protein losses due to binding (see Materials and Methods), the avoidance of pooled samples which introduced massive impurities, and a brisk routine to obtain data before the enzyme lost activity. We thus avoided pitfalls which led to separation of the wrong protein (41).

The subunit molecular weight determined by SDS-gel electrophoresis was 56,000. The protein smeared on gel filtration chromatography, presumably due to interaction of the extremely hydrophobic protein with the stationary phase, and we assume a homodimeric native protein (Table 2). The N-terminal amino acids were determined to be XSTVLYRXPEL.

Cloning, sequencing, and identification of the *tsaMBCD* and *tsaR* genes. We made a restriction digest map of the *tsaM* locus (Fig. 4) with the aid of an oligonucleotide probe representing the N terminus of TsaM (as indicated in Fig. 5) and confirmed the map with a probe representing the internal amino acid sequence (see above). Total DNA was subjected to digestion with *Sal*I and *Bgl*II, and fragments in the 4-kb region, localized in a Southern blot with a probe for *tsaM*, were ligated into pBluescript-KSII. Clones (250) were screened with the probe for *tsaM*, and a single clone was identified. Both DNA strands of the 4-kb *Sal*I-*Bgl*II insert were partially sequenced. One complete (*tsaR*, see below) and one incomplete open reading frame (ORF) were detected. The incomplete ORF included the N-terminal and internal sequences from the monooxygenase component, TsaM. To complete this sequence, a 6-kb *Kpn*I fragment (Fig. 4), which overlaps the 4-kb *Sal*I-*Bgl*II fragment, was cloned and partially sequenced. The 6-kb fragment was identified with a 0.3-kb *Kpn*I-*Bgl*II probe derived from the 4-kb *Sal*I-*Bgl*II clone. Downstream of *tsaM* we located a complete ORF, whose deduced sequence includes a section identical to the N-terminal sequence of reductase TsaB. In total, 6,040 bp were sequenced and five ORFs were found and analyzed (Fig. 4 and 5).

Sequence and characterization of monooxygenase TsaMB. The start codon (ATG) of monooxygenase *tsaM* was found at position 1477 (Fig. 5); the stop codon (TGA) was found at position 2518. We located a putative Shine-Dalgarno sequence (AAGGAG, position 1464) upstream of this gene (68). The start codon of reductase *tsaB* was found at position 2517, the stop codon was found at position 3468, and a putative Shine-Dalgarno sequence (CGGGA) was found at nucleotide 2505. The stop codon of *tsaM* (TGA) and the start codon of *tsaB* (ATG) overlap (Fig. 5). Gene *tsaM* has a coding capacity for 347 amino acids, giving a calculated molecular weight ($M_{r,calc}$) of 39,557, and gene *tsaB* has a coding capacity for 317 amino acids ($M_{r,calc}$ = 34,186); these molecular weights corresponded to the values (43,000 and 36,000, respectively) obtained with SDS-PAGE.

A sequence analysis of TsaM confirmed the presence of motifs attributed to a Rieske [2Fe-2S] center (e.g., Cys-48,

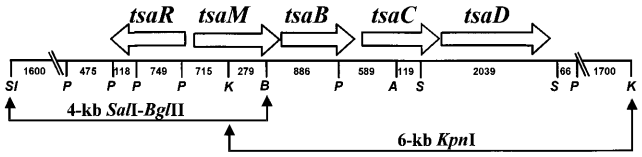


FIG. 4. Restriction digest map of the region containing operon *tsaMBCD* and *tsaR*. The two clones (*Sal*I-*Bgl*II and *Kpn*I) used for sequencing are shown. Only the restriction sites used for subcloning are shown. Distances between two restriction sites are marked in base pairs. The orientation and location of *tsaM*, *tsaB*, *tsaC*, *tsaD*, and *tsaR* are shown with arrows. *Sl*, *Sal*I; *P*, *Pst*I; *K*, *Kpn*I; *B*, *Bgl*II; *A*, *Acc*I; *S*, *Sac*I.

Cys-67, His-50, and His-70) and a mononuclear iron center (Asp-155, His-158, and His-163) (Fig. 5) (see also references 44 and 46) and confirmed the enzyme as a member of the class IA oxygenases (Table 3).

Similarly, TsaB was confirmed as a typical class IA reductase (Table 3) with presumed sites for *proR* NADH binding (60) (Gly-117, Gly-118, Ile-119, Gly-120, Thr-122, Pro-123, Met-127, Arg-145, Pro-195, Phe-232), flavin mononucleotide binding (Arg-51, Tyr-53, Ser-54, Leu-55), and a ferredoxin [2Fe-2S] center (Cys-266, Cys-271, Cys-274, Cys-304) (Fig. 5; see also references 44 and 46).

Sequencing and characterization of *p*-sulfobenzyl alcohol dehydrogenase TsaC. Downstream of reductase gene *tsaB* (Fig. 4 and 5) we located an ORF (759 bp) termed *tsaC*. The stop codon of reductase *tsaB* and the start codon of *tsaC* were 13 bp apart. The start codon of *tsaC* (ATG) was found at position 3484, the stop codon (TGA) was found at position 4240, and a potential Shine-Dalgarno sequence (AACCA) was found at position 3473. The deduced amino acid sequence (Fig. 5) includes the N-terminal sequence determined for the *p*-sulfobenzyl alcohol dehydrogenase (Fig. 1), and the $M_{r,\mathrm{calc}}$ (26,438) corresponds to the observed value (29,000); *tsaC* thus encodes the alcohol dehydrogenase. TsaC has a high degree of sequence similarity (Table 3) with group II, short-chain, NAD(P)-dependent, zinc-independent alcohol dehydrogenases (54) for which a crystal structure is available (25). The conserved residues Asp-62 (Fig. 5), Ser-142, Tyr-155, and Lys-159 may be involved in the catalytic mechanism, whereas the pattern Gly-12–XXX–Gly-16–X–Gly-18 (part of the αβα motif) and Thr-11 of the N terminus presumably represent conserved amino acids in the coenzyme binding domain. These data confirm the assumption (41) of the classification of this enzyme.

Sequencing and characterization of *p*-sulfobenzaldehyde dehydrogenase TsaD. The stop codon of *tsaC* was found to overlap the start codon (ATG, position 4239) of a 1,427-bp ORF, termed *tsaD*, with a potential Shine-Dalgarno sequence (ACGGG) at position 4223 and a stop codon (TGA) at position 5667. The deduced amino acid sequence (Fig. 5) includes the N-terminal sequence determined for the aldehyde dehydrogenase (Fig. 1), and $M_{r,\mathrm{calc}}$ (51,010) corresponds to the observed value (56,000); *tsaD* thus encodes the *p*-sulfobenzaldehyde dehydrogenase. A data bank analysis showed high levels of similarity to a wide variety of aldehyde dehydrogenases (Table 3). The N-terminal region is poorly conserved, but a number of strictly conserved amino acids were found, especially Gly residues which are distributed throughout the protein. The residues Cys-286 (Fig. 5) and Gly-230 to Gly-235 (αβα motif) may be part of the NAD binding fold and Glu-252 is thought to be part of the active site (32). However, this interpretation conflicts with another (30), and no crystal structure is available to resolve the disagreement.

The region downstream of *tsaMBCD*. No ORF was found downstream (370 bp) of *tsaMBCD*. A putative transcription stop signal [stem-loop and poly(T) sequence] in the region from 5688 to 5718 and the functionality of the upstream region (see below) indicate that this gene cluster represents an operon.

Sequencing of the putative LysR-type transcriptional regulator, *tsaR*. At 114 bp upstream of *tsaM* an ORF, termed *tsaR*, was found in the reverse orientation to *tsaMBCD*. The putative start codon (GTG) of *tsaR* was attributed to position 1362, the stop codon (TGA) was attributed to position 468, and a potential Shine-Dalgarno sequence (CGGGA) was attributed to position 1374. A sequence analysis showed that *tsaR* would encode a protein containing 298 amino acids ($M_{r,\mathrm{calc}}$ = 32,630) with similarities, largely at the N terminus (where the helix-turn-helix motif for DNA binding was located), to LysR-type transcriptional regulators (Table 3; Fig. 5 [where only highly conserved residues of the αβα structure are marked]) (59).

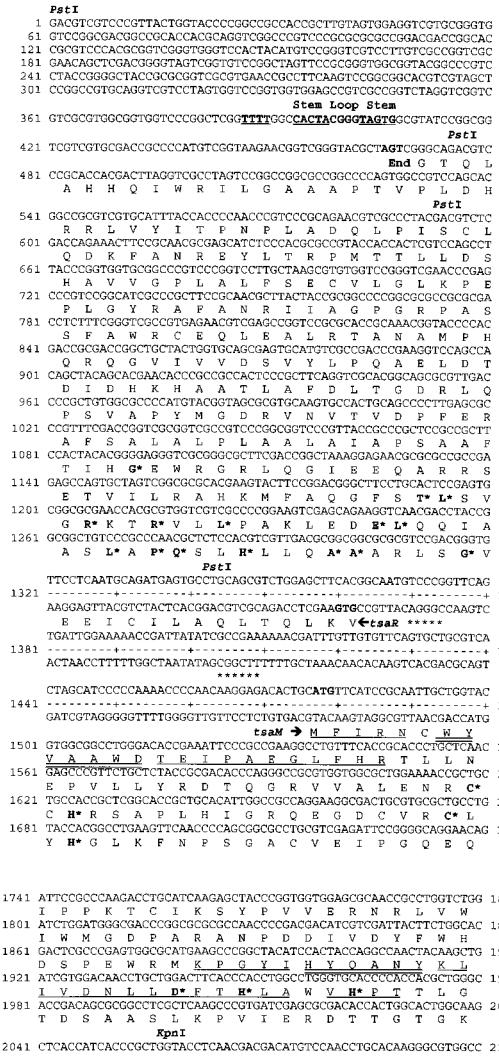
The region downstream of *tsaR*. No ORF was found downstream (465 bp in the direction of transcription) of *tsaR*. A putative transcription stop signal [stem-loop and poly(T) sequence] in the region from 387 to 407 and the location of the *tsaMBCD* operon indicate that this single gene constitutes a separate transcriptional unit.

Structure of the region between *tsaMBCD* and *tsaR*. The intergenic region between *tsaR* and *tsaM*, 114 bp, was found to have a GC content of 48%, much lower than the 70% observed in the coding regions. A low GC content of the intergenic region has been observed previously between a LysR-type regulator gene and the neighboring gene(s) it regulates (77). One putative recognition site for a LysR-type regulator was found, position 1376 (TTC-8 bp-GAA), which corresponds to Schell's model (59). A putative sigma^{70} "housekeeping" promoter of operon *tsaMBCD* is located upstream from *tsaM*, though the Pribnow box TAGCAT at position 1442 and the −35 region (1418; TTGTTG) show only weak homologies with the consensus sequences (45). Another potential promoter, which could be recognized by a sigma^{70} factor, lies upstream of *tsaR*: the Pribnow box (position 1401; TATAA; reverse strand) is identical to the consensus, whereas the −35 region (position 1424; ACAACA; reverse strand) has poor homology with the consensus sequence.

DISCUSSION

Each of the genes in the cluster *tsaMBCD* has been identified from the deduced N-terminal amino acid sequence (Fig. 5) and by its size. This cluster thus encodes the inducible enzymes which catalyze the reactions (Fig. 1) of the physiologically defined regulatory unit R1 involved in the degradation of toluenesulfonate in *C. testosteroni* T-2 (61). The evidence that the cluster represents a four-gene operon lies in the absence of neighboring genes (in the direction of transcription (Fig. 5), in the putative stop signal (Fig. 5), and in the detection of a 4.5-kb mRNA (33a), i.e., a polycistronic messenger of the correct length.

We presume that the putative LysR-type regulatory gene, *tsaR*, is expressed as a one-gene transcriptional unit and that its product is involved in both autoregulation and regulation of expression of the neighboring *tsa* catabolic operon. *tsaR* is typical of the supergene family, both from its structure and from the nature of the intergenic region with which it would have to interact (59), but only experiments will confirm this hypothesis. The induction of operons controlled by LysR-type regulators takes place through the binding to that protein of a substrate or an intermediate of the pathway, so toluenesulfon-



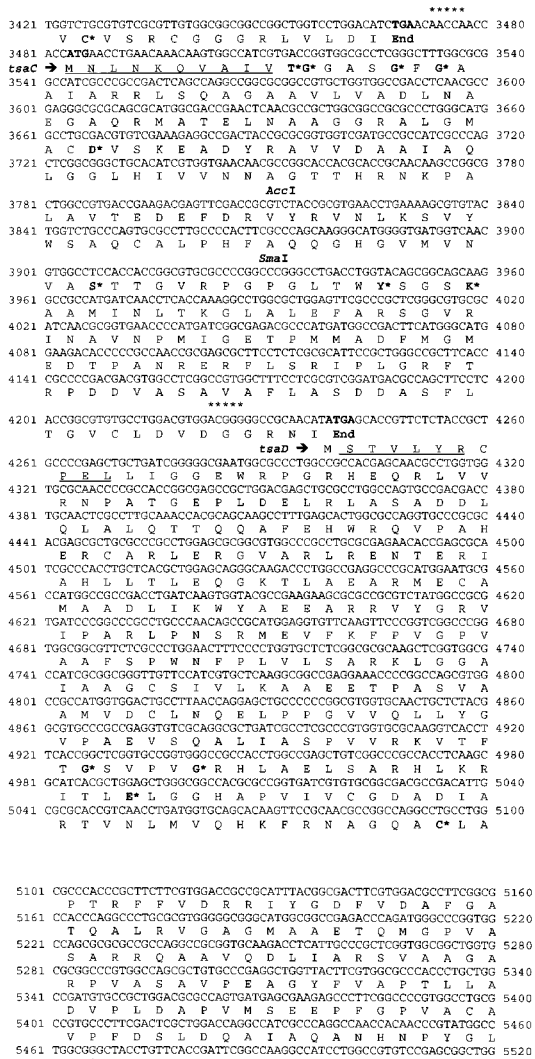


FIG. 5. Nucleotide sequences of the toluenesulfonate operon *tsaMBCD* and of its putative regulator gene *tsaR*. Positions 1 to 1362, reverse strand; positions 1321 to 1500, double strand; positions 1501 to 6040, sense strand. Putative regulator *tsaR* (positions 1362 to 469, reverse strand), methyl-monooxygenase *tsaM* (positions 1477 to 2517), reductase *tsaB* (positions 2517 to 3467), alcohol dehydrogenase *tsaC* (positions 3484 to 4239), and aldehyde dehydrogenase *tsaD* (positions 4239 to 5666) are shown. Start codons and stop codons are in boldface letters. The chemically sequenced N-terminal and internal amino acids are underlined, and the amino acids used to deduce the oligonucleotide probes are doubly underlined. Putative transcriptional stop signals [poly(T) nucleotides and stem-loops] are underlined and in boldface. Restriction sites used for subcloning are also shown. Putative Shine-Dalgarno sequences are indicated by a lightface asterisk. Selected, conserved amino acids are in boldface with a boldface asterisk.

TABLE 3. Comparison of TsaMBCD and TsaR with related proteins

Protein	$M_{r,calc}$[a]	Structure	Position		GC content (%)	Related proteins[b]					
			SD[c]	Gene		Name	% Identity	$M_{r,calc}$	Structure	GC content (%)	Accession no.
TsaR	32,630 (298)	ND[d]	1374	1362–469	70	Nac	27.8	32,754 (305)	ND	62	Q08597
						ClcR	27.2	32,527 (294)	ND	56	L06464
						SdsB	27.1	32,955 (306)	ND	74	M86744
						CynR	26.9	32,961 (299)	ND	55	P27111
						TfdR	25.9	32,070 (295)	ND	67	P10086
TsaM	39,557 (347)	Tetramer	1464	1477–2517	66	VanA	32.7	36,578 (329)	ND	69	P12609
						Pht3	23.8	49,297 (439)	Tetramer	59	D13229
						CbaA	23.7	48,927 (432)	ND	59	U00692
						PobA	21.0	46,258 (409)	ND	67	X78823
TsaB	34,186 (317)	Monomer	2505	2517–3467	71	VanB	41.2	33,706 (314)	ND	73	P12580
						PobB	38.9	33,558 (319)	ND	73	X78823
						Pht2	34.1	35,999 (324)	Monomer	57	D13229
						CbaB	30.9	31,699 (288)	ND	59	U00692
TsaC	26,438 (252)	Dimer	3473	3484–4239	69	Xan	38.9	26,143 (250)	ND	69	S47054
						BudC	37.4	25,313 (241)	Tetramer	63	Q04520
						3Bhd	37.4	26,848 (253)	ND	59	P19871
						LinX	36.8	25,492 (250)	ND	59	D23722
						Dhk1	36.1	28,393 (272)	ND	72	P16542
TsaD	51,010 (476)	Dimer?	4223	4239–5666	71	GabD	38.7	51,719 (482)	ND	58	P25526
						AldH	34.7	54,158 (497)	ND	54	X95396
						Ald	34.0	52,142 (478)	Tetramer	51	P25553
						DhaC	33.7	54,743 (500)	Dimer	ND	P15437
						AldH6	33.3	56,009 (512)	ND	55	A55684

[a] $M_{r,calc}$, molecular weight calculated from amino acid composition. The total number of amino acids is in parentheses.
[b] LysR-type regulators: Nac, nitrogen assimilation control regulator; CynR, cyanate operon regulator; SdsB, sodium dodecyl sulfatase regulator; ClcR, chlorocatechol operon regulator; TfdR, 2,4-dichlorophenoxyacetate operon regulator. Oxygenases and reductases: VanAB, Pht23, CbaAB, and PobAB (see legend for Fig. 6). Group II dehydrogenases (DH): Xan, hypothetical protein; BudC, acetoin (diacetyl) DH; 3Bhd, 3-β-hydroxysteroid DH; LinX, γ-hexachlorocyclohexane dehydrochlorinase; Dhk1, putative ketoacyl reductase (granaticin polyketide synthase). Aldehyde DHs: GabD, succinate-semialdehyde DH; AldH, aldehyde DH; Ald, lactaldehyde DH; DhaC, horse cytosol aldehyde DH; AldH6, human cytosol aldehyde DH.
[c] SD, Shine-Dalgarno sequence.
[d] ND, not determined.

ate or toluenecarboxylate or the intermediates in the pathway (Fig. 1) could interact with TsaR. The LysR-type autoregulatory transcriptional regulators are widespread and regulate many target genes, which include catabolic operons for xenobiotics (13, 75). A subfamily of regulators for chlorocatechol degradation has been described (62) that shows homologies to TsaR (see Table 3). LysR-type regulators are often plasmid encoded and involved in the positive regulation of catabolic operons. TfdR (Table 3) is located on plasmid pJP4, which belongs to the same incompatibility group, IncP1β, as plasmid pTSA (see below) and has a similar GC content.

The genes we have sequenced (Fig. 5) encode regulatory and catalytic functions. We know that transport is essential to the metabolism of sulfonates, both theoretically, because these charged compounds would otherwise not come in contact with the soluble cytoplasmic enzymes catalyzing their degradation, and from direct observation of a toluenesulfonate transport system in strain T-2 (42). The inducible transport system must thus be encoded at another locus, and it will be interesting to discover whether TsaR also regulates its expression. The enzymes encoded by the *tsa* catabolic operon also convert *p*-toluenecarboxylate to terephthalate when the organism utilizes toluate (41). It is unclear whether a toluenecarboxylate transport system is required for growth by strain T-2, but when at least porins are required for the metabolism of xylene (XylN) and benzene (27a, 79), it seems likely that some membrane structure will be required by the organism to utilize toluenecarboxylate.

The GC content of total DNA of *C. testosteroni* T-2 is 61.8% ± 0.5%, typical of the species (10). Chromosomally located genes in *C. testosteroni* have a GC content of 61% ± 1% with 73% for the third base (1, 2, 8, 11, 20, 36, 50). In contrast, our ORFs show a high GC content (70%) and an extremely high GC content in the third base (91%). The codon usage among the genes in both the *tsa* operon and *tsaR* is the same but different from that of the chromosomal genes. We have now shown that the *tsa* operon and *tsaR* are located on plasmid pTSA, and we conclude that these genes were recruited from another organism (33b).

Aerobic metabolism of inert aromatic compounds typically starts by oxygenation at the ring or at the (methyl) side chain (see reference 41 and citations therein). Although many ring oxygenations have been studied, our understanding of oxygenation of the methyl side chain is limited largely to work related to the TOL plasmids, and even there, the oxygenase XylM has not been purified (70). The work with *C. testosteroni* T-2 thus presents the first set of data in which all catabolic enzymes involved in side chain manipulation have been purified, characterized, and sequenced. By comparison of the TOL and TSA systems, we see clearly that nature has used very different detailed solutions for oxidizing a methyl group by the "same" pathway. The TOL upper pathway has a different gene order

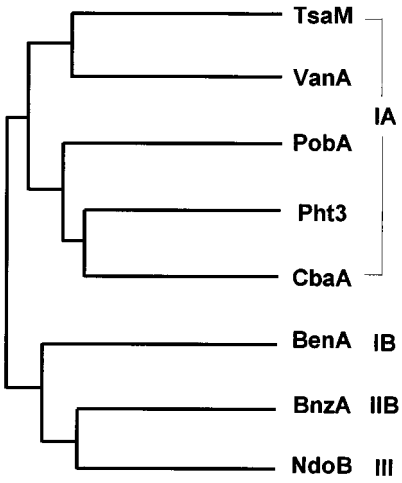


FIG. 6. Rooted evolutionary tree of class IA multicomponent oxygenases with representatives from classes IB, IIB, and III based on the amino acid composition of the oxygenases. VanA, vanillate demethylase (9); CbaA, 3-chlorobenzoate 2,3-dioxygenase (46); Pht3, phthalate 4,5-dioxygenase (49); PobA, phenoxybenzoate dioxygenase (15); BenA, benzoate dioxygenase (accession number [AC.] P07767); BnzA, benzene dioxygenase (AC. P08084); NdoB, naphthalene dioxygenase (AC. P23094). The N-terminal amino acid sequence of *p*-sulfobenzoate dioxygenase PsbA (another dioxygenase system in the degradative pathway of toluenesulfonate) (39) displays a high sequence identity value of 48% with CbaA over the available 35-amino-acid sequence, indicating that the oxygenases are closely related (46) and that PsbAC is another class IA dioxygenase system. *p*-Methoxybenzoate demethylase system MbdAB (no sequence data available) apparently shares substrates with vanillate-demethylase system VanAB (3-methoxybenzoate and 3,4-dimethoxybenzoate) and monooxygenase system TsaMB (4-methylbenzoate and 4-methoxybenzoate), whereas TsaMB does not share any common substrate with VanAB. We expect high levels of sequence similarity between monooxygenase MbdAB and the other two monooxygenases.

than the *tsa* catabolic operon and includes a transport function (XylN); different regulatory systems are used (sigma^{54} promoter, integration host factor, and the NtrC-like regulator XylR in the TOL system contrasted with putative sigma^{70} promoters and LysR-type regulation) and different types of monooxygenases and different gene families of alcohol dehydrogenases (class I in TOL [67] and class II in *tsa* [Table 3]) are involved. Only the aldehyde dehydrogenases (Table 3) (see the introduction) belong to the same family, with 29% sequence identity between TsaD and XylC being determined. When two types of attack on a methyl side chain differ so markedly (see also reference 31), we may expect yet more diversity when further pathways of oxidation of aromatic methyl groups are examined.

The short-chain alcohol dehydrogenases have been found frequently in aromatic metabolism, but usually as diene-diol dehydrogenases (e.g., XylL) (47), which represent a very different reaction type (regeneration of the aromatic ring structure) from that of TsaC (Fig. 1). Sequence comparisons similar to those in Table 3 show that the diene-diol dehydrogenases have identities with TsaC lower than 32% (Table 3).

As anticipated from biochemical data (44), monooxygenase system TsaMB has a high level of homology to class IA oxygenase systems, the reductases showing a higher level of identity than the oxygenases (Table 3). We generated an evolutionary tree for oxygenase components of oxygenase systems with an arbitrarily set root (Fig. 6). It was apparent that, despite the similarities in class IA, there are two separate evolutionary lines in this group of oxygenase components. The subdivision of the class IA oxygenase components into monooxygenases and dioxygenases, considered with the similarity of the Rieske centers (Table 4) (27), presumably indicates differences due to the binding site for the aromatic substrate and oxygen. In contrast, no subgroups were apparent in trees obtained from alignments of sequences from reductase components (not shown). It is well known that the domain structure of the reductases in class IA differs from those in other classes. Thus, we rationalize the homogeneity in the class IA reductases in the common property of supplying electrons to the oxygenase components.

Whereas there is considerable understanding of the function of the reductases of the mononuclear iron oxygenase systems (14, 22, 44) and a steady increase in our understanding of the [2Fe-2S] Rieske center in the oxygenase (27, 28, 44), there is still very little information on the mononuclear iron itself. The long-standing data from Bernhardt's group (7), from which no sequence data are available, are only now being expanded (72). The close biochemical similarity of monooxygenase TsaMB and *p*-methoxybenzoate demethylase (M_r, redox centers, amino acid composition, and overlapping substrate ranges [e.g., 4-methoxybenzoate and 4-methylbenzoate]) (40) serves to link the biophysical work (7) with the biochemical and/or sequencing work done with several dioxygenase systems (Table 3).

The catalytic enzymes (TsaM, TsaC, and TsaD) encoded by the *tsa* operon do not discriminate between the sulfonate and the carboxyl substituents, whereas the subsequent enzymes do (61). Despite the similarities of both aromatic substituents, it seems that their different physical and chemical properties sometimes require specialized enzymes. Each class IA oxygenase requires an aromatic carboxyl or, as in this case, an aromatic sulfonate substituent in its substrate. As monooxygenase TsaM can accommodate a sulfonate substituent, the question of whether other class IA oxygenases can tolerate a sulfonate substituent and still oxygenate the compound arises.

The high degree of similarity of monooxygenase and dioxygenase systems seen in Fig. 6 (51), in the face of old strictures

TABLE 4. Data from EPR spectra of the [2Fe-2S] centers in the oxygenase components of some multicomponent oxygenase systems

Enzyme	Class	g_z	g_y	g_x	Center	Source or reference
4-Toluenesulfonate methyl-monooxygenase	IA	2.019	1.918	1.790	Rieske	This paper
4-Sulfobenzoate DOS[a]	IA	2.025	1.921	1.745	Rieske	35
4-Methoxybenzoate demethylase	IA	2.008	1.913	1.72	Rieske	73
Phthalate DOS	IA	2.016	1.914	1.763	Rieske	12
4-Chlorophenylacetate DOS	IA	2.021	1.922	1.737	Rieske	43
2-Halobenzoate DOS	IB	2.025	1.912	1.788	Rieske	19
Pyrazon DOS	IIA	2.02	1.91	1.79	Rieske	58
Benzene DOS	IIB	2.018	1.917	1.754	Rieske	23
Naphthalene DOS	III	2.01	1.91	1.80	Rieske	69

[a] DOS, dioxygenase system.

from the Enzyme Commission (81), is not restricted to sequence comparisons. Not only can dioxygenase systems monooxygenate some substrates (26, 78), but a monooxygenase system (MbdAB) can dioxygenate at least one substrate (82). When promulgated, the wording of the Enzyme Commission rule was quite correct (29). It would seem appropriate to adjust the rules of nomenclature to the biochemical and molecular biological facts, especially as a monooxygenase system in class IB has also been detected (56).

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